ORIGINAL ARTICLE

Evaluation of high-resolution melting analysis for *spa*-typing of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates

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Abstract

Molecular characterization of *Staphylococcus aureus* isolates from nosocomial and community-acquired infections using accurate, reproducible, and rapid typing methods is essential for the fast identification of prevalent and epidemic strains. Although sequence-based *spa* typing is highly effective, PCR-based techniques (such as high-resolution melting curve analysis, HRM) are simpler, less expensive, faster, and can be performed in a single and closed-tube assay format, thereby reducing the risk of contamination. A total of 51 methicillin-resistant *S. aureus* (MRSA) (n = 26) and methicillin-sensitive *S. aureus* (MSSA) (n = 25) isolates from Karaj (n = 10) and Yasuj (n = 41), Iran, were subjected to HRM. All selected isolates were identified by the standard *spa*-typing method. Among the 51 tested isolates, 11 genotype profiles were distinguished from 12 *spa* types. Strains t1077 and t1816 exhibited the highest and lowest melting temperatures (81.8°C and 79.4°C), with 46.7% and 39.8% G + C contents, respectively. Strains t706 and t1816, with almost identical G + C contents, had the same HRM genotypes, but their curves differed due to different G + C distributions. Four standard *spa* types (strains t030, t037, t701 and t5598) were differentiated correctly and their melting temperatures were 81.2°C, 81.4°C, 80.4°C and 80.1°C, respectively. We demonstrated that HRM profiling is a rapid method which enables the accurate screening of certain strains (especially the endemic ones), and may be used for bacterial surveillance. However, it cannot replace sequence-based *spa* typing, especially for newly emerging *spa* types, and therefore cannot be used as a standardized global method.

Keywords: High-resolution melting analysis, HRM, Iran, MRSA, MSSA, *spa* type
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Introduction

Methicillin-resistant *Staphylococcus aureus* (commonly known as MRSA) was first detected in the early 1960s. To date, MRSAs have been identified as major pathogens, especially in healthcare-associated infections (HA-MRSA), and are considered as major threats to human health [1–4]. The emergence of community-acquired infections caused by highly pathogenic MRSA clones (CAMRSA for community-acquired MRSA) has challenged the management of infections [5,6]. In order to identify rapidly spreading strains, the use of molecular typing methods is vital; such methods are essential for the active surveillance and control of disease outbreaks. Hence, to investigate outbreaks and improve epidemiological studies, the molecular characterization of MRSA clones is a crucial prerequisite [3].

Widely used techniques for MRSA typing include multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and sequence-based typing of the variable X region of the staphylococcal protein A gene (*spa* typing) [6–8]. The polymorphic X region of the staphylococcal protein A (*spa*) gene can develop rapidly, and diversity patterns within sequence types can be explained by *spa* sequence typing [9]. *Spa* typing has been used as a

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single-locus sequence genotyping [8,10,11] to study the local and global epidemiology of *S. aureus* infections [3,12]. Despite the complexity and high cost of PCR sequencing [3,13], PCR-based typing has still remained as an efficient and reasonable approach [6,14]. However, real-time PCR (as a non-sequence-based genotyping method) offers the possibility of obtaining results at the time of diagnosis and thus improves infection management [11]. A recent approach following the development of real-time PCR is *spa* typing based on high-resolution melting (HRM) [11,15,16], a rapid, cost-effective, and robust method for strain differentiation based on single-nucleotide polymorphism (SNP) [3,6,11,13,16,17]. It has been demonstrated to be extremely effective for distinguishing *S. aureus spa* types [9,11].

The major feature of HRM is alteration of the melting behavior of double-stranded DNA; this is achieved by sequence variations within a DNA fragment which subsequently yields a specific melting curve profile [6,18]. Very small temperature increments are applied to achieve accurate melting curves. It is very important to know whether various amplicons have the same or different sequences; this is possible through the normalization and comparison of the obtained melting curves [16,19]. Since HRM differentiates the melting curves of different amplicons based on their shape, even if the same Tm values are obtained, this method is potentially much more powerful than conventional melting curve analysis [11].

Here, we investigated 51 MRSA and MSSA *S. aureus* strains by HRM-based *spa* typing to discriminate them from one another and to test the reliability and efficiency of this typing method. To the best of our knowledge, this is the first attempt to investigate both methicillin-resistant and -susceptible *S. aureus* strains by this method.

Methods

MRSA and MSSA isolates

A total of 51 *S. aureus* isolates—including 26 MRSA and 25 MSSA strains from Karaj (ten MRSA) and Yasuj (16 MRSA and 25 MSSA), Iran—were evaluated by HRM analysis. All selected isolates were identified by the standard *spa*-typing method as previously published (t5598, t2684, t969, t1149, t706, t692, t030, t037, t1816, t701, t6871, t1077) [20,21].

DNA extraction

Briefly, 1 mL of overnight culture of *S. aureus* on trypticase soy broth (TSB; BD, Germany), was centrifuged at 5000 × *g* for 5 min. The supernatant was removed and 300 µL Tris–EDTA (TE) buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 100 µL lysozyme (25 mg/mL) were added to the pellet. After vortexing, the suspension was incubated at 37°C. After 60–75 min, 3–4 µL proteinase K (100 g/mL; Sigma) and 150 µL SDS 10% were added. The suspension was then incubated for 60–75 min at 37°C. Boiling water was used to heat the suspension for 10 min; the suspension was then cooled rapidly on ice. The lysate was extracted using the phenol–chloroform extraction method as previously described [20]. Nanodrop and agarose electrophoresis gels (1.5% wt/vol) were used to measure the concentration and quality of the purified DNAs. Finally, the extracted DNAs were stored at −20°C for future use.

High-resolution melting (HRM) for *spa*-typing

Using the 5x HOT FIREPol EvaGreen HRM Mix (Solis Bio-Dyne Co, Estonia), the polymorphic X region of the *spa* gene was amplified in a Rotor-Gene Q instrument (Qiagen) using HRM analysis. Briefly, a 20-mL PCR reaction was prepared, containing 4 mL 5x HOT FIREPol EvaGreen HRM Mix, 0.5 mL of each primer (according to http://www.ridom.de/spaserver/), 14 mL double-distilled water (ddH_2O), and 1 mL of the template DNA. The real-time PCR reaction condition was as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s. The temperature range for high-resolution melting analysis of the amplicons was between 75°C and 95°C with stepwise increases of 0.1°C.

High Resolution Melt Software v3.0 (Thermo Fisher Scientific Inc., USA) was used to analyse the melting curves of all samples. Reactions were carried out in triplicate, and the DNAs from four known *spa* types (t030, t037, t701 and t5598) were used as standard melting curves.

Results

All 12 *spa* types identified among the 51 *S. aureus* isolates were differentiated by Tm except for two strains. Each distinct *spa* type exhibited a different HRM profile except the t706 and t692 strains which had the same HRM type. Overall, 11 HRM profiles were distinguished among the 12 *spa* types. The melting temperatures (Tm, °C) and G + C contents of the 12 *spa* types are presented in Table 1. *Spa* types t706 and t1816 had the same HRM genotype but the shapes of their curves were different. The four standard *spa* types (t030, t037, t701 and t5598) were differentiated accurately with respective melting temperatures of 81.2°C, 81.4°C, 80.4°C and 80.1°C.

Discussion

The high frequency of *S. aureus* in the healthcare settings of Iran has caused concerns about the increasing incidence of infections associated with this microorganism. Prevalent strains

TABLE 1. Results of 51 clinical isolates from Karaj and Yasuj, Iran, undergoing *spa* typing by applying high-resolution melting analysis (HRM) and conventional PCR sequencing methods

spa type (n = 12)	No. of isolates (n = 51)	PCR-based *spa* repeat units	HRM type (n = 12)	Mean Tm, °C	Repeat region GC%	Repeat region size, bp
t5598 (MSSA)	3	08-16-34-24-34-17	1	80.1	43	144
t2684 (MSSA)	2	09-13-16-13-17-34-16-34	2	79.8	42.2	192
t969 (MRSA)	2	15-12-02-24-24	3	80.9	45	120
t1149 (MSSA)	3	08-16-34-24-34-17-17	4	80.6	44	168
t706 (MRSA)	4	09-02-16-34-17-34-16-34	5	79.7	41.7	192
t692 (MSSA)	3	07-12-21-17-34-34-34-34-33-34	5	79.5	40	240
t030 (MRSA)	9	15-12-16-02-24-24	6	81.2	45.1	144
t037 (MRSA)	11	15-12-16-02-25-17-24	7	81.4	45.2	168
t1816 (MSSA)	2	07-12-21-17-34-13-34-34-34-33-34	8	79.5	39.8	264
t701 (MSSA)	5	11-10-21-17-34-24-34-22-25-25	9	80.4	43.3	240
t1077 (MSSA)	4	14-44-12-17-23-18-17	10	81.8	46.7	165
t6871 (MSSA)	3	15-12-16-02-12-16-02-16-02-25-17-24-24	11	81.0	43.9	312

can be identified rapidly through the nationwide molecular characterization of MRSA and MSSA isolates causing nosocomial and community-acquired infections. This is feasible through a precise, reproducible, and rapid typing method [14]. Due to the cost and limited intra- and inter-laboratory reproducibility of pulsed-field gel electrophoresis (PFGE) as the reference standard method for the typing of *S. aureus* isolates, researchers decided to replace it with multilocus sequence typing (MLST) and staphylococcal protein A (*spa*) typing methods [10].

Spa typing is very useful for *S. aureus*. It exhibits an excellent discriminatory power, high reproducibility, cost effectiveness, and speed in both local and global investigations. However, despite the high efficiency of sequence-based *spa* typing, sequence-free PCR-based techniques such as HRM are simpler and cheaper. HRM is fast and has the advantage of being performed in a single closed tube, thereby reducing the risk of contamination. HRM has been recognized as a suitable candidate for replacing *spa* typing owing to its speed, cost effectiveness, suitability for high-throughput screening, reproducibility, and simplicity [6,19].

Although initial HRM results showed some similar melting temperatures and curve shapes, by optimizing several methods and materials—such as DNA extraction method and HRM Master mix—a real-time PCR-based HRM assay was developed to differentiate *S. aureus* isolates, and especially major MRSA *spa* types (t037 and t030) present in Iran. In agreement with previous studies [3,6,9,11,19], a standardized HRM *spa* typing can determine the various *spa* sequence alleles. In the present study, 11 HRM profiles were distinguished among 12 *spa* types, including four standard *spa* types (t030, t037, t701, and t5598). According to the current and previous studies, the results of HRM for *spa* typing can be affected by various assay parameters such as the real-time thermal cycler (Rotor-Gene Q instrument, Qiagen, used in this study), HRM software, DNA quality, DNA and primer concentrations and HRM master mix [19]. This may explain why the results (especially the melting temperatures) may differ even for a given strain. For example, the melting temperatures of strains exhibiting the t030 and t037 *spa* types differed in different studies [3,11,13,19]. As an example, the Tm obtained for *spa* type t037 varied depending on the HRM master mix [3,11].

The real-time thermal cycler and HRM master mix are the factors that can most significantly affect the HRM results. In the present study we used a Rotor-Gene Q instrument (Qiagen) and the 5x HOT FIREPol EvaGreen HRM Mix (ROX) (Solis BioDyne Co, Estonia) for HRM analysis, while previous studies used different thermal cyclers such as LightCycler Nano real-time PCR and different HRM master mixes including Platinum SYBR-Green qPCR Super Mix-UDG (Life Technologies), and SensiMix HRM (Bioline) mix with Eva-Green dye [3,9,11,13,19]. Moreover, in agreement with the findings of other studies [3,11,13,19], it seems that HRM results are mostly affected by the G + C content of tested strains rather than their size bands.

As mentioned in the results section of this article, the highest Tm (81.8°C) was for t1077 which also had the highest G + C content (46.7%), while the lowest Tm (79.4°C) was observed for t1816 which had the lowest G + C content (39.8%). The four standard *spa* types (t030, t037, t701 and t5598) with different G + C contents were accurately discriminated. Although t706 and t1816, with nearly identical G + C contents, had the same HRM genotype, they were differentiated by the HRM curve analysis owing to different composition and distribution of GC.

With the standardization and improvements of the HRM method carried out in this study, some predominant *spa* types in Iran—in particular t030 and t037—could be differentiated. Since several factors (band size, G + C content, real-time thermal cycler, HRM software, DNA and primer concentrations, and DNA quality) can affect HRM results, this method can be used as a screening method along with other genotyping methods to increase the discrimination power in molecular

typing, reduce the expense, and minimize the risk of sample contamination. HRM curve profiling is fast, can accurately screen certain strains (especially endemic ones), and can be used for the surveillance of predominant strains. However, it may not replace sequence-based *spa* typing, especially for newly emerging *spa* types.

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Conflict of interest

None declared.

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